

To: Mary Schiflett
Program Administrator
TMC/NASA Cooperative Research Program
406 Jesse H. Jones Library Building
T: 791-8844; F: 791-8855

Round One
FINAL REPORT

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Round 1 Progress Report: Anabolic Vitamin D Analogs as Countermeasures to Bone Loss

Norman J. Karin¹, Mary C. Farach-Carson², Co-PIs

¹Univ. Texas Grad Sch. Biomed. Sci., GSBS S.102, T: 500-9870; F: 500-9877

²Dept. Basic Sciences, Univ. Texas Dental Branch, DBB 3.108, T: 500-4492; F: 500-4500

During the month of June, 1997, the final month of this contract, Dr. Wei Li continued his investigation of the "priming" effect of vitamin D₃ on parathyroid hormone (PTH)-induced elevations in cytosolic free Ca²⁺ in cultured osteoblasts. Unlike the vitamin D steroid, neither estrogen nor progesterone were either to prime the cells' response to PTH. Wei also confirmed previous experimental results suggesting that vitamin D₃ alone does not change cytoplasmic Ca²⁺ levels.

To summarize the progress we made during the period supported by the Round 1 contract, we demonstrated for the first time that vitamin D₃ influences the effect of PTH on bone cell calcium ion levels. This is a rapid effect, taking place within seconds/minutes. This may prove to be a critical contribution to our understanding of bone physiology in that these two hormones are among the most potent regulators of bone calcium content and of systemic calcium homeostasis. Together with the data gathered from the study of astronauts exposed to microgravity for extended periods, these observations suggest the interaction of vitamin D₃ and PTH as a possible therapeutic target in the treatment of bone loss disorders such as osteoporosis and disuse atrophy. Our findings have been accepted for publication (copy attached):

Li, W., Karin, N.J. and Farach-Carson, M.C. (1997). 1,25-Dihydroxyvitamin D₃ Enhances Parathyroid Hormone-Induced Increase in Cytosolic Free Calcium in Osteoblastic Cells through a Membrane-Initiated Pathway. Am. J. Physiol. (in press).

Another result from the Round 1 study was our observation that chronic exposure of cultured osteoblasts to vitamin D₃ altered the number of voltage-sensitive Ca²⁺ channels expressed. Estrogen treatment yielded a similar result, suggesting that there is overlap in the mechanism by which these hormones elicit long-term effects on bone cell calcium homeostasis.

1,25(OH)₂D₃ enhances PTH-induced Ca²⁺ transients in preosteoblasts by activating L-type Ca²⁺ channels

WEI LI,¹ RANDALL L. DUNCAN,² NORMAN J. KARIN,¹ AND MARY C. FARACH-CARSON¹

¹Department of Basic Science, University of Texas-Houston, Dental Branch, Houston, Texas 77030; and ²Department of Orthopaedic Surgery, Indiana University Medical Center, Indianapolis, Indiana 46202

Li, Wei, Randall L. Duncan, Norman J. Karin, and Mary C. Farach-Carson. 1,25(OH)₂D₃ enhances PTH-induced Ca²⁺ transients in preosteoblasts by activating L-type Ca²⁺ channels. *Am. J. Physiol.* 273 (Endocrinol. Metab. 36): E599–E605, 1997.—We previously demonstrated electrophysiologically that 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] shifts the activation threshold of L-type Ca²⁺ channels in osteoblasts toward the resting potential and prolongs mean open time. Presently, we used single-cell Ca²⁺ imaging to study the combined effects of 1,25(OH)₂D₃ and parathyroid hormone (PTH) during generation of Ca²⁺ transients in fura 2-loaded MC3T3-E1 cells. Pretreatment with 1,25(OH)₂D₃ concentrations, which alone did not produce Ca²⁺ transients, consistently enhanced Ca²⁺ responses to PTH. Enhancement was dose dependent over the range of 1 to 10 nM and was blocked by pretreatment with 5 μM nitrendipine during pretreatment. A 1,25(OH)₂D₃ analog that activates L-type channels and shifts their activation threshold also enhanced PTH responses. In contrast, an analog devoid of membrane Ca²⁺ effects did not enhance PTH-induced Ca²⁺ transients. The PTH-induced Ca²⁺ transient involved activation of a dihydropyridine-insensitive cation channel that was inhibited by Gd³⁺. Together, these data suggest that 1,25(OH)₂D₃ increases osteoblast responsiveness to PTH through rapid modification of L-type Ca²⁺ channel gating properties, whose activation enhances Ca²⁺ entry through other channels such as the PTH-responsive, Gd³⁺-sensitive cation channel.

calcitropic hormones; vitamin D; bone cells; calcium homeostasis; parathyroid hormone; 1,25-dihydroxyvitamin D₃

BOTH 1,25-DIHYDROXYVITAMIN D₃ [1,25(OH)₂D₃] and parathyroid hormone (PTH) play fundamental roles in controlling bone density and systemic Ca²⁺ homeostasis. Bone is a major target tissue for PTH and 1,25(OH)₂D₃, and cells of the osteoblastic lineage possess receptors for and respond to both hormones (6, 26). The secosteroid 1,25(OH)₂D₃ activates both genomic and nongenomic (membrane-initiated) pathways in osteoblastic cells, presumably through separate receptor systems (23). The nuclear vitamin D receptor for 1,25(OH)₂D₃ (nVDR) is well characterized and has been the subject of numerous comprehensive reviews (21). In contrast, the identity of the membrane receptor and its action in controlling intracellular events remain elusive (22). Our laboratory previously demonstrated, using electrophysiological recording techniques, that 1,25(OH)₂D₃ increases osteoblastic plasma membrane permeability to Ca²⁺ by shifting the threshold of L-type Ca²⁺ channel activation toward the resting potential and prolonging the channel mean open time (4). This phenomenon occurs within milliseconds after addition

of 1,25(OH)₂D₃. These observations led us to hypothesize that 1,25(OH)₂D₃ exerts a “priming” effect on membrane-initiated Ca²⁺ responses to other calcitropic hormones acting through plasma membrane receptors, such as PTH (13). These responses generally are coupled to Ca²⁺ release from intracellular stores and to nonselective cation channels in the plasma membrane and together produce transient elevations in cytosolic free Ca²⁺ (12). An enhancing effect of 1,25(OH)₂D₃ would be predicted to augment Ca²⁺ signaling in response to other calcitropic hormones and could be manifested as an increase in either the magnitude or duration of the Ca²⁺ transient.

Synthetic analogs of 1,25(OH)₂D₃ containing various structural modifications can stimulate subsets of biological activities in target cells. These analogs have been characterized extensively in several laboratories, including our own (reviewed in Ref. 1). Analogs such as 1,24-dihydroxy-22-ene-24-cyclopropyl D₃ (code name BT, also known as calcipotriol) bind well to the nVDR and selectively activate genomic pathways such as those that lead to increased transcription of bone matrix proteins such as osteopontin and osteocalcin (14). Unlike the parent hormone, 1,25(OH)₂D₃, these analogs produce little or no acute stimulation of Ca²⁺ influx at low nanomolar concentrations (14). Other analogs, in particular those lacking the 1-α-hydroxyl group, such as analogs 25-hydroxy-16-ene-23-yne-D₃ (code name AT) and 25-hydroxy-23-yne-D₃ (code name Y), lack the ability to bind to the nVDR or initiate transcription of matrix proteins but readily increase Ca²⁺ influx into osteoblastic cells (16). These latter analogs also shift the activation threshold for L-type Ca²⁺ channels toward the resting membrane potential (30). We postulated that these readily distinguishable activities reflect a pharmacological distinction between the two receptors for 1,25(OH)₂D₃, one nuclear and one in or near the plasma membrane (14).

In this study, we used a single-cell Ca²⁺ imaging system to examine the interaction of 1,25(OH)₂D₃ or two of the previously characterized 1,25(OH)₂D₃ analogs, AT and BT, with PTH. Specifically, we examined the potential Ca²⁺-enhancing effect of the secosteroids with regard to increases in the concentration of free intracellular Ca²⁺ ([Ca²⁺]_i) induced by PTH in preosteoblastic MC3T3-E1 cells loaded with fura 2. A nonfusing, premycytic cell line, BC₃H₁, which expresses 1,25(OH)₂D₃-responsive L-type plasma membrane Ca²⁺ channels at high levels characteristic of excitable tissues (3, 10), was studied for comparative purposes. The role of Ca²⁺ influx through voltage-sensitive Ca²⁺ channels present in the plasma membrane in the

1,25(OH)₂D₃-enhancement phenomenon was demonstrated using inhibitors of channel function. Interaction with the Gd³⁺-inhibitable, mechanosensitive cation channel found in osteoblasts (12) was also revealed for the first time.

MATERIALS AND METHODS

Materials. Coverslip tissue culture dishes were obtained from MatTek (Ashland, MA). Fura 2-AM, the acetoxymethyl ester of the Ca²⁺-sensitive fluorescent dye, fura 2, was purchased from Molecular Probes (Eugene, OR). Thapsigargin was obtained from Calbiochem (La Jolla, CA). Bovine PTH-(1–34), nitrendipine, Gd³⁺, and other chemicals were purchased from Sigma Chemical (St. Louis, MO). 1,25(OH)₂D₃ was from Biomol Research Laboratories (Plymouth Meeting, PA), and structural analogs were kindly provided by Dr. Anthony Norman (University of California at Riverside, Riverside, CA).

Cell culture. MC3T3-E1 cells, a preosteoblastic line derived from neonatal mouse calvarial bone, were provided by Dr. Renny Franceschi and maintained as culture stocks in ascorbate-free medium containing 10% fetal bovine serum, as described previously (15). Growth phase BC₃H₁ premyocytes were cultured in Dulbecco's modified Eagle's medium (DMEM)-Ham's F12 (1:1) medium containing 10% fetal bovine serum as described (25). Cells were plated onto coverslip dishes in DMEM containing 10% fetal bovine serum 2 days before the day of the experiment. All cells were subconfluent at the time of the experiments.

Intracellular Ca²⁺ measurements. We used a single-cell Ca²⁺ imaging system (Intracellular Imaging, Cincinnati, OH) to perform intracellular Ca²⁺ measurements (28). After the medium was removed from the dishes, cells were rinsed with Hanks' balanced salt solution (HBSS) (140 mM NaCl, 4.2 mM KCl, 0.5 mM NaH₂PO₄, 0.4 mM Na₂HPO₄, 0.4 mM MgSO₄, 0.3 mM MgCl₂, 1 mM CaCl₂, 6 mM glucose, 0.1% bovine serum albumin, and 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, pH 7.4) and then loaded with 3 μM fura 2-AM in HBSS for 30 min at 37°C. The conditions were chosen to avoid probe compartmentalization and to maximize cytoplasmic dye localization. The loaded cells were incubated further for 15 min with HBSS alone to allow the complete deesterification of fluorescent probe. Fura 2 fluorescence was visualized with a Nikon inverted microscope using a Nikon ×40 fluor objective. The cells were illuminated with a xenon lamp equipped with quartz collector lenses. A shutter and filter changer containing the two different interference filters (340 and 380 nm) were computer controlled. Emitted light was passed through a 430-nm dichroic mirror, filtered at 510 nm, and imaged with an integrating charge-coupled device video camera. Four to eight cells were measured within each field. Consecutive frames obtained at 340- and 380-nm excitation were compared as a ratio (F₃₄₀/F₃₈₀), and [Ca²⁺]_i in each cell was calculated from F₃₄₀/F₃₈₀ by comparison with fura 2 free acid standards. Individual Ca²⁺ traces shown in Figs. 1–4 are computer-generated population means derived from simultaneous recording of [Ca²⁺]_i in the four to eight single cells in a microscopic field. Each experiment was repeated at least three times, and Figs. 1–4 were constructed from representative experiments.

1,25(OH)₂D₃ and structural analogs. 1,25(OH)₂D₃ and structural analogs AT and BT were stored as stock solutions in absolute ethanol in the dark at –20°C until use. The structural integrity and concentrations of the compounds were routinely monitored from the absorption spectra and by comparison of the absorbency ratio at 264/228 nm, as de-

scribed previously (4). Solutions with a ratio <1.6 were discarded.

Other methods. Bovine PTH-(1–34) was dissolved in distilled water. Thapsigargin and nitrendipine were maintained and dispensed from stock solutions in dimethyl sulfoxide (DMSO) or absolute ethanol, respectively. All reagents were stored in the dark at –20°C. The delivery vehicle was used as the control in all experiments.

RESULTS

1,25(OH)₂D₃ enhances PTH-induced increases in cytosolic Ca²⁺ concentration in preosteoblastic MC3T3-E1 cells. In our initial experiments, we measured the ability of nanomolar concentrations of 1,25(OH)₂D₃ to induce transient rises in [Ca²⁺]_i in single cells representing the preosteoblastic and premyocytic phenotypes, both previously shown to express L-type Ca²⁺ channels responsive to secosteroids (4, 10, 20). As shown in Fig. 1, exogenously added 1,25(OH)₂D₃ (10 nM) produced an immediate and rapid increase of [Ca²⁺]_i in fura-loaded premyocytic BC₃H₁ cells (Fig. 1A) but no significant [Ca²⁺]_i increase in preosteoblastic MC3T3-E1 cells (Fig.

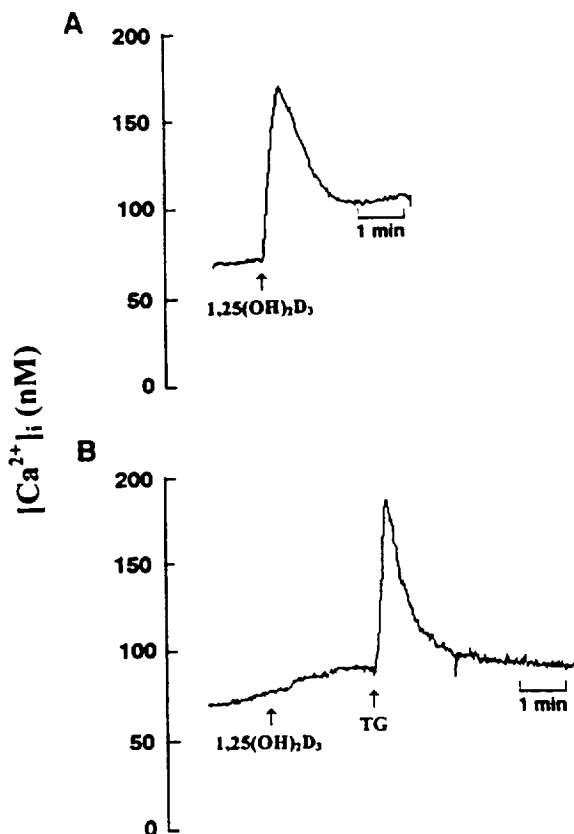


Fig. 1. Effect of 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] on concentration of free intracellular Ca²⁺ ([Ca²⁺]_i) in growth phase myocytic BC₃H₁ cells and preosteoblastic MC3T3-E1 cells. Measurements of [Ca²⁺]_i were made using a single-cell Ca²⁺ imaging system, as described in MATERIALS AND METHODS. A: BC₃H₁ cells treated with 10 nM 1,25(OH)₂D₃ demonstrate a Ca²⁺ transient that peaks within 15 s and then rests at new baseline. B: MC3T3-E1 cells treated with same concentration of 1,25(OH)₂D₃ do not show a similar Ca²⁺ transient but will release Ca²⁺ from intracellular stores in response to 5 μM thapsigargin (TG). First arrow denotes time of addition of 1,25(OH)₂D₃.

1B). The lack of response in MC3T3-E1 cells is not attributable to an absence of releasable Ca²⁺ in intracellular stores, because addition of thapsigargin immediately produced a Ca²⁺ transient (Fig. 1B). Depolarization of the MC3T3-E1 cells with 60–120 mM extracellular K⁺ also failed to elicit a Ca²⁺ signal detected by fura 2, although ⁴⁵Ca²⁺ influx studies showed that depolarization triggered an increase in Ca²⁺ uptake within 2 min (data not shown). We next examined the potential ability of 1,25(OH)₂D₃ to enhance PTH effects on [Ca²⁺]_i in MC3T3-E1 preosteoblasts, a direct test of our hypothesis that the left shift in activation potential toward the resting potential would augment development of the Ca²⁺ transient induced by PTH. The traces presented in Fig. 2 show that in MC3T3-E1 cells, pretreatment with 10 nM 1,25(OH)₂D₃ for 10 min before PTH stimulation (Fig. 2A) enhanced the PTH-induced Ca²⁺ transient compared with control pretreatment with vehicle (ethanol) alone (Fig. 2B). To investigate if influx through L-type Ca²⁺ channels is required for the enhancement effect, we tested whether inclusion of 5 μM nitrendipine, a dihydropyridine blocker of L-type Ca²⁺ channels, would attenuate the enhancement effect of 1,25(OH)₂D₃. As seen in Fig. 2C, the Ca²⁺ transient induced by PTH after treatment with both 1,25(OH)₂D₃ and nitrendipine was comparable to that produced by PTH in cells treated with vehicle alone (compare Fig. 2, B and C). Because nitrendipine did not block the PTH-induced Ca²⁺ transient (Fig. 2C), we tested whether this transient could be blocked by Gd³⁺, a lanthanide cation that

inhibits stretch-activated Ca²⁺-conducting channels known to be present in osteoblasts (12). As shown in Fig. 2D, addition of 10 μM Gd³⁺ completely abolished the PTH-induced Ca²⁺ transient, even after addition of 1,25(OH)₂D₃. The same effect was seen if the Gd³⁺ was added during the pretreatment period (data not shown). Even when intracellular stores were full (Fig. 1B), the elimination of extracellular Ca²⁺ completely abolished development of the Ca²⁺ transient after addition of 1,25(OH)₂D₃ and PTH, indicating that influx of extracellular Ca²⁺ was required for development of the Ca²⁺ signal in response to PTH (data not shown). The increased slope after addition of PTH was a common, but not invariable, occurrence during repetition of these experiments. At present we have no explanation for this phenomenon. In further studies, we tested whether the enhancing effect on PTH-induced influx through Gd³⁺-sensitive channels produced by 1,25(OH)₂D₃ was dose dependent. An enhancement of the PTH-induced increase in [Ca²⁺]_i by pretreatment with 5 nM 1,25(OH)₂D₃ was seen, although the magnitude of [Ca²⁺]_i rise was less than that induced by pretreatment with the 10-nM dosage (compare Fig. 3, A and B). Treatment with 1 nM 1,25(OH)₂D₃ produced a barely detectable enhancement of PTH-induced Ca²⁺ signals (Fig. 3C). Simultaneous addition of 1,25(OH)₂D₃ and PTH did not increase the magnitude or duration of the Ca²⁺ transient (data not shown) relative to that induced by PTH alone.

BC₃H₁ premyocytes did not exhibit Ca²⁺ transients in response to the addition of PTH (data not shown),

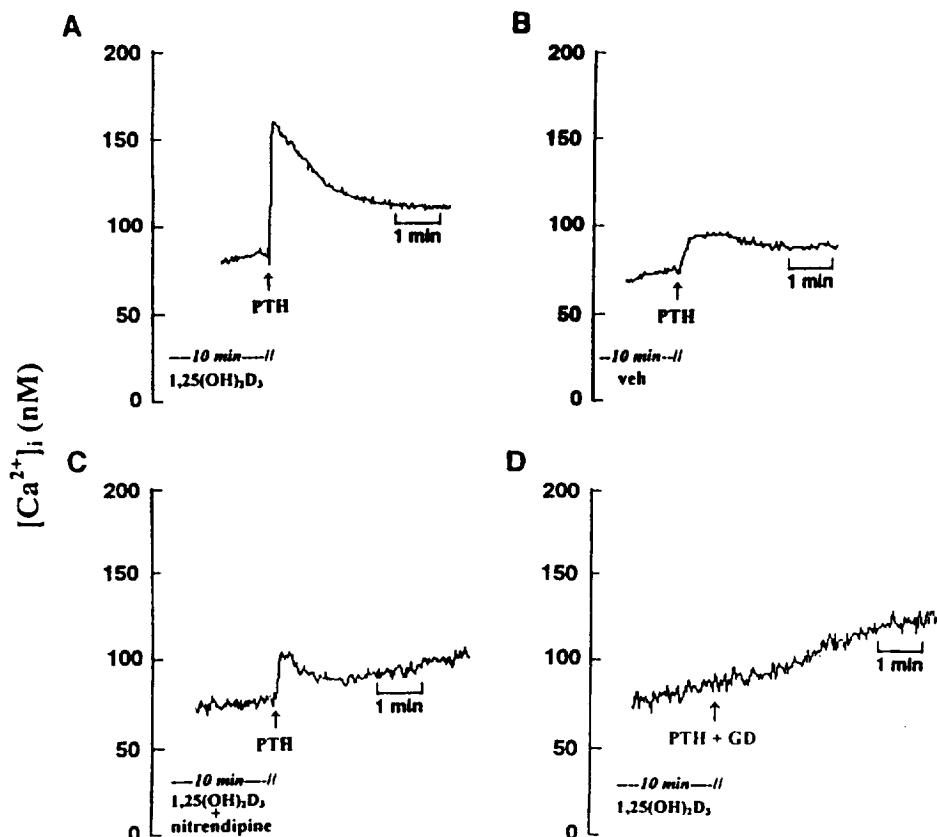


Fig. 2. Enhancement of parathyroid hormone (PTH)-stimulated increase in [Ca²⁺]_i by 1,25(OH)₂D₃. MC3T3-E1 cells loaded with fura 2 were pretreated with 10 nM 1,25(OH)₂D₃ (A), ethanol vehicle (B), or 10 nM 1,25(OH)₂D₃ + 5 μM nitrendipine (C) for 10 min, at the end of which time PTH (0.5 μM) was added (arrows). Comparison of Ca²⁺ transient produced in A and B demonstrates enhancement effect, which is blocked by inclusion of nitrendipine (C). Even after 10 min of pretreatment with 1,25(OH)₂D₃, PTH-induced Ca²⁺ transient is completely blocked if Gd³⁺ (GD; 10 μM) is added (D). This PTH-induced transient is not blocked by nitrendipine (C).

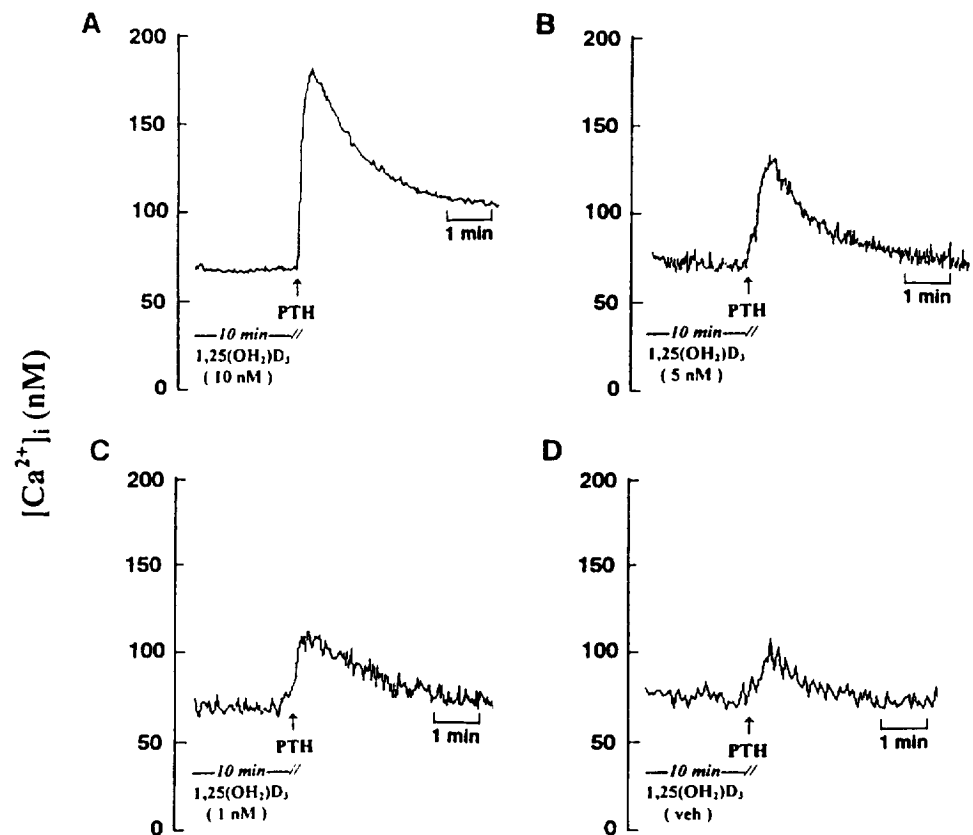


Fig. 3. Ability of 1,25(OH)₂D₃ to enhance PTH-induced Ca²⁺ currents is dose dependent. MC3T3-E1 cells were treated with various concentrations of 1,25(OH)₂D₃ for 10 min, then PTH (0.5 μM) was added. Enhancement of PTH-induced Ca²⁺ signal produced by 1,25(OH)₂D₃ was dose dependent between 1–10 nM. As shown in Fig. 1B, this concentration of 1,25(OH)₂D₃ alone did not produce a Ca²⁺ transient. A, 10 nM 1,25(OH)₂D₃; B, 5 nM 1,25(OH)₂D₃; C, 1 nM 1,25(OH)₂D₃; D, vehicle alone.

presumably because they lack appropriate PTH-responsive receptor systems.

Enhancement of the Ca²⁺ transient stimulated by PTH by pretreatment with analog AT but not analog BT. We used two analogs of 1,25(OH)₂D₃ that selectively activate either genomic nVDR-mediated (analog BT) or nongenomic membrane-initiated Ca²⁺-signaling pathways (analog AT) in osteoblastic cells (14). We tested the ability of analogs AT and BT to augment the PTH-induced rise in $[Ca^{2+}]_i$ in MC3T3-E1 preosteoblasts, analogous to the previous experiments using 1,25(OH)₂D₃. Pretreatment of cells with analog AT (10 nM) for 10 min enhanced the Ca²⁺ transient produced by PTH (Fig. 4A), which was not seen with the vehicle control (Fig. 2B). This enhancement by analog AT was similar in magnitude and duration to that obtained with the parent compound, 1,25(OH)₂D₃. Conversely, pretreatment with analog BT (10 nM) had no effect on the increase in $[Ca^{2+}]_i$ induced by PTH (Fig. 4B). Neither AT nor BT directly produced a rise in $[Ca^{2+}]_i$ in MC3T3-E1 cells in the absence of PTH (data not shown). The inclusion of nitrendipine with analog AT completely negated the enhancement effect (Fig. 4C). Inclusion of Gd³⁺ (Fig. 4D) also completely abolished the response to PTH after pretreatment with analog AT.

DISCUSSION

1,25(OH)₂D₃ and PTH are calcitropic hormones that potentiate long-term regulation of bone structure and physiology. This control is exerted, at least in part, by

osteoblasts that contain specific receptors for these circulating hormones (6, 26). In the complex processes of bone remodeling and Ca²⁺ homeostasis, the effects of each hormone alone and in combination on the activity of osteoblasts must be considered. In addition to the well-characterized genomic actions attributed to activation of nVDRs, 1,25(OH)₂D₃ also produces rapid changes in membrane Ca²⁺ permeability that are independent of hormonal regulation of gene expression (8, 16). In this regard, changes in osteoblastic $[Ca^{2+}]_i$ might serve as signals to regulate systemic Ca²⁺ homeostasis by modulating transfer of soluble bone Ca²⁺ to the general extracellular fluid.

Addition of PTH to primary cultures of osteoblasts (17) or to clonal osteoblast-like osteosarcoma cell lines (11, 27, 29) elicits a rapid but transient elevation of $[Ca^{2+}]_i$ that is generated by influx of Ca²⁺ through plasma membrane channels coupled to release of Ca²⁺ from intracellular stores. Furthermore, proliferating cultures of MC3T3-E1 cells express mRNA encoding the PTH receptor, the levels of which increase during cell differentiation (18). In differentiated osteoblasts, 1,25(OH)₂D₃ also induces rapid increases in $[Ca^{2+}]_i$ by stimulation of transmembrane influx combined with release of Ca²⁺ from intracellular stores (8, 17). Ca²⁺ influx in response to calcitropic agents can be blocked by polyvalent transition metal cations and by several organic Ca²⁺ channel antagonists (8, 17) and thus involves voltage-gated Ca²⁺ channels. Previous studies have also demonstrated that osteoblastic cells express Gd³⁺-sensitive, stretch-activated channels that can be

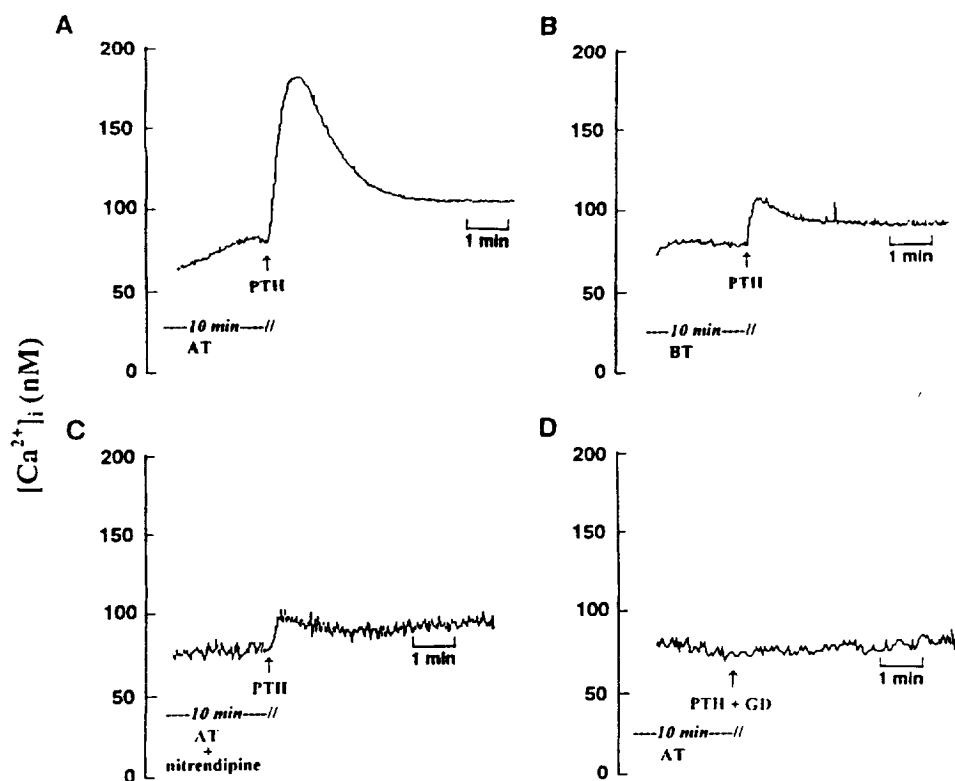


Fig. 4. Enhancement of PTH-stimulated increase in $[Ca^{2+}]_i$ by analog 25-hydroxy-16-ene-23-yne-D₃ (AT) but not analog 1,24-dihydroxy-22-ene-24-cyclopropyl D₃ (BT). MC3T3-E1 cells loaded with fura 2 were pretreated with 10 nM analog AT (A) or analog BT (B) or 10 nM AT + 5 μ M nitrendipine (C), after which time PTH (0.5 μ M) was added (arrows). Effect of AT but not BT was similar to that of 1,25(OH)₂D₃. AT-enhanced, PTH-induced transient was completely abolished in presence of 10 μ M Gd³⁺ (D).

stimulated by PTH and provide a large capacitative entry of extracellular Ca²⁺ (12).

In this study, neither 1,25(OH)₂D₃ treatment (10 nM) nor K⁺ depolarization significantly increased $[Ca^{2+}]_i$ in preosteoblastic MC3T3-E1 cells loaded with fura 2, even though intracellular stores were filled. In contrast, an immediate and rapid increase in $[Ca^{2+}]_i$ was produced by addition of 1,25(OH)₂D₃ to BC₃H₁ premyocytic cells. In previous studies, single-channel measurements performed using a patch clamp revealed that osteosarcoma cells possess about $1-2 \times 10^3$ functional L-type Ca²⁺ channels per cell (4). In comparison, differentiated clonal BC₃H₁ myocytes express $1-2 \times 10^4$ Ca²⁺ channels per cell (2, 25). The very different density of functional Ca²⁺ channels in the plasma membranes of BC₃H₁ myocytes and MC3T3-E1 cells may account for the differences in the response of these two cell types to 1,25(OH)₂D₃ that we report in Fig. 1. These observations are consistent with the origin of these cell lines in tissues considered to be "excitable" and "nonexcitable," respectively, in which only the former are believed to possess the machinery involved in Ca²⁺-induced Ca²⁺ release from intracellular stores (9). Supporting this, we previously reported that UMR-106 osteosarcoma cells have no detectable Ca²⁺-induced Ca²⁺ release (19), assessed by insensitivity to treatment with caffeine. In multiple experiments with fura 2-loaded cells, we were unable to detect transient increases in $[Ca^{2+}]_i$ in proliferating MC3T3-E1 cells treated with 1,25(OH)₂D₃ alone or subjected to K⁺ depolarization. In contrast, Oshima et al. (24) reported transient elevations in $[Ca^{2+}]_i$ in response to 1,25(OH)₂D₃ but not 24,25(OH)₂D₃. We believe that this difference reflects a greater state of

differentiation in their MC3T3-E1 cultures, which were first grown to confluence, subcultured, then withdrawn from the cell cycle by transfer to low serum medium. We previously found that the steady-state levels of mRNA-encoding L-type Ca²⁺ channels in MC3T3-E1 cells increase substantially during differentiation (20), and it is possible that other systems involved in Ca²⁺-induced release of Ca²⁺ from stores or regulating Ca²⁺ influx are similarly upregulated.

Earlier studies showed that cells derived from neonatal rat calvaria possess two classes of voltage-gated Ca²⁺ channels of the "low threshold" (T-type) and "high threshold" (L-type) (7), the latter identified by their sensitivity to organic Ca²⁺ channel antagonists, in particular the dihydropyridines. In a previous study (4), our laboratory demonstrated, using single channel recording techniques, that osteoblastic osteosarcoma cells express L-type but not T-type Ca²⁺ channels that respond both to dihydropyridine agonists, such as BAY K 8644, and antagonists, such as nitrendipine. Additionally, we found that, within milliseconds of addition of 1,25(OH)₂D₃, there consistently occurred a shift in the threshold of activation of inward L-type Ca²⁺ currents to more negative and near-resting potentials, which single-channel analysis revealed was accompanied by a prolonged open time of individual channels (4). On the basis of these findings, we predicted that this shift in the activation threshold would result in an increased responsiveness to other calcitropic hormones, such as PTH, that also activate plasma membrane Ca²⁺ influx through voltage-insensitive channels (12, 13). In this report, we show that in MC3T3-E1 cells, PTH alone stimulates only a modest increase in $[Ca^{2+}]_i$. However,

pretreatment with 1,25(OH)₂D₃ for 10 min dramatically enhanced the PTH-induced Ca²⁺ transient, clearly indicating that 1,25(OH)₂D₃ served a priming function to enhance Ca²⁺ responsiveness at the level of the plasma membrane. The need for preincubation with 1,25(OH)₂D₃ suggests the existence of intracellular pathways involving second messengers, which take minutes to transmit the signal to the PTH-response system. The block of activation by removal of extracellular Ca²⁺ or addition of dihydropyridine channel blockers indicates that enhancement of the PTH response absolutely depends on the presence and influx of extracellular Ca²⁺ through L-type channels.

We previously measured the ability of structural analogs of 1,25(OH)₂D₃ to stimulate various genomic and plasma membrane-initiated events (14). Although 1,25(OH)₂D₃ functions as the natural ligand for initiation of both long-term and rapid responses in target cells, we identified subsets of response pathways that were activated by discrete structural analogs. Analog AT activates Ca²⁺ channels in plasma membranes without binding to nVDRs, whereas analog BT binds the nVDR for 1,25(OH)₂D₃ without triggering a measurable influx of extracellular Ca²⁺. We examined the effects of analogs AT and BT alone and in combination with PTH on regulation of [Ca²⁺]_i in MC3T3-E1 cells. Neither AT nor BT alone increased [Ca²⁺]_i in MC3T3-E1 cells. However, pretreatment with analog AT enhanced the transient rise in [Ca²⁺]_i stimulated by PTH, consistent with the shift in the threshold of L-type channel activation toward the resting potential ("left shift") reported for this analog in previous studies (30). Unlike analog AT, analog BT did not enhance the PTH-induced elevation in [Ca²⁺]_i. This also is consistent with the inability of this nVDR-selective analog to produce a left shift in the activation threshold at the low nanomolar concentrations used in these studies (30).

Taken together, these data strongly support our hypothesis that 1,25(OH)₂D₃ and Ca²⁺-activating analogs serve a priming function by activating plasma membrane voltage-sensitive Ca²⁺ channels. One interpretation of these findings is that voltage-sensitive Ca²⁺ channel activation is required for subsequent full activation of the Gd²⁺-sensitive channel by PTH, since nitrendipine addition eliminated the enhancement of PTH-sensitive Ca²⁺ influx. A second possibility is that the Gd³⁺-sensitive channel and the L-type channel must both be activated to generate sufficient depolarization of the plasma membrane to permit development of a large Ca²⁺ transient. In either case, these data indicate the existence of an additional level of interaction of these hormones separate from the previously characterized genomic regulatory loops (5). We believe that this previously unappreciated and novel action of 1,25(OH)₂D₃ may facilitate the action of other hormones and growth factors acting on osteoblasts and could explain some of the seemingly contradictory physiological effects of 1,25(OH)₂D₃. It will be of interest to elucidate the mechanism by which Ca²⁺ influx through L-type channels in the plasma membrane is linked to release of Ca²⁺ from intracellular Ca²⁺ stores

in osteoblasts and to influx through voltage-insensitive Ca²⁺ channels.

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Address for reprint requests: M. C. Farach-Carson, Dept. of Basic Science, Univ. of Texas-Houston, Dental Branch, Houston, TX 77030.

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REFERENCES

1. Bouillon, R., W. H. Okamura, and A. W. Norman. Structure-function relationships in the vitamin D endocrine system. *Endocr. Rev.* 16: 200–257, 1995.
2. Caffrey, J. M., A. M. Brown, and M. D. Schneider. Mitogens and oncogenes can block the induction of specific voltage-gated ion channels. *Science* 236: 570–573, 1987.
3. Caffrey, J. M., and M. C. Farach. A monoclonal antibody specifically modulates dihydropyridine-sensitive calcium current in BC3H1 myocytes. *Mol. Pharmacol.* 34: 518–526, 1988.
4. Caffrey, J. M., and M. C. Farach-Carson. Vitamin D₃ metabolites modulate dihydropyridine-sensitive calcium currents in clonal rat osteosarcoma cells. *J. Biol. Chem.* 264: 20265–20274, 1989.
5. Cantley, L. K., J. Russell, D. Lettieri, and L. M. Sherwood. 1,25-Dihydroxyvitamin D₃ suppresses parathyroid hormone secretion from bovine parathyroid cells in tissue culture. *Endocrinology* 117: 2114–2119, 1985.
6. Chen, T. L., M. A. Hirst, and D. Feldman. A receptor-like binding macromolecule for 1,25-dihydroxycholecalciferol in cultured mouse bone cells. *J. Biol. Chem.* 254: 7491–7494, 1979.
7. Chesnoy-Marchais, D., and J. Fritsch. Voltage-gated sodium and calcium currents in rat osteoblasts. *J. Physiol. (Lond.)* 398: 291–311, 1988.
8. Civitelli, R., Y. E. Kim, S. L. Gunsten, A. Fujimori, M. Huskey, L. V. Avioli, and K. A. Hruska. Nongenomic activation of the calcium message system by vitamin D₃ metabolites in osteoblast-like cells. *Endocrinology* 127: 2253–2262, 1990.
9. Clapham, D. E. Calcium signalling. *Cell* 80: 259–268, 1995.
10. deBoland, A. R., and R. L. Boland. Non-genomic signal transduction pathway of vitamin D in muscle. *Cell. Signal.* 6: 717–724, 1994.
11. Donahue, H. J., M. J. Fryer, E. F. Eriksen, and H. Heath. Differential effects of parathyroid hormone and its analogues on cytosolic calcium ion and cAMP levels in cultured rat osteoblast-like cells. *J. Biol. Chem.* 263: 13522–13527, 1988.
12. Duncan, R. L., K. A. Hruska, and S. Misler. Parathyroid hormone activation of stretch-activated cation channels in osteosarcoma cells (UMR-106). *FEBS Lett.* 307: 219–223, 1992.
13. Farach-Carson, M. C., and S. E. Guggino. Organ-specific actions of vitamin D analogs: relevance of rapid effects. In: *Organ Selective Actions of Steroid Hormones, Ernst Schering Research Foundation Workshop 16*, edited by D. T. Baird, G. Schutz, and R. Krattenmacher. Berlin: Springer-Verlag, 1995, p. 161–180.
14. Farach-Carson, M. C., I. Sergeev, and A. W. Norman. Nongenomic activation of 1,25-dihydroxyvitamin D₃ in osteosarcoma cells: structure-function studies using ligand analogs. *Endocrinology* 129: 1876–1884, 1992.
15. Franceschi, R. T., and B. S. Iyer. Relationship between collagen synthesis and expression of the osteoblast phenotype in MC3T3-E1 cells. *J. Bone Miner. Res.* 7: 235–246, 1992.
16. Khoury, R., A. L. Ridall, A. W. Norman, and M. C. Farach-Carson. Target gene activation by 1,25-dihydroxyvitamin D₃ in osteosarcoma cells is independent of calcium influx. *Endocrinology* 135: 2446–2453, 1994.

17. Lieberherr, M. Effects of vitamin D₃ metabolites on cytosolic calcium in confluent mouse osteoblasts. *J. Biol. Chem.* 262: 13168–13173, 1987.
18. McCauley, L. K., A. J. Koh, C. A. Beecher, Y. Cui, J. D. Decker, and R. T. Franceschi. Effects of differentiation and transforming growth factor β 1 on PTH/PTHrP receptor mRNA levels in MC3T3-E1 cells. *J. Bone Miner. Res.* 10: 1243–1255, 1995.
19. Meszaros, J. G., and N. J. Karin. Inhibitors of ER Ca²⁺-ATPase activity deplete the ATP- and thrombin-sensitive Ca²⁺ pool in UMR 106–01 osteosarcoma cells. *J. Bone Miner. Res.* 10: 704–710, 1995.
20. Meszaros, J. G., N. J. Karin, and M. C. Farach-Carson. Voltage-sensitive calcium channels in osteoblasts: mediators of plasma membrane signalling events. *Connect. Tissue Res.* 35: 161–165, 1996.
21. Minghetti, P. P., and A. W. Norman. 1,25(OH)₂D₃ receptors: gene regulation, and genetic circuitry. *FASEB J.* 2: 3043–3053, 1988.
22. Nemere, I., M. C. Dormanen, M. W. Hammond, W. H. Okamura, and A. W. Norman. Identification of a specific binding protein for 1 α ,25-dihydroxyvitamin D₃ in basal-lateral membranes of chick intestinal epithelium and relationship to transcaltachia. *J. Biol. Chem.* 269: 23750–23756, 1994.
23. Norman, A. W., I. Nemere, L.-X. Zhou, J. E. Bishop, E. E. Lowe, A. C. Maiyar, E. D. Collins, T. Taoka, I. Sergeev, and M. C. Farach-Carson. 1,25(OH)₂ vitamin D₃, a steroid hormone that produces biologic effects via both genomic and nongenomic pathways. *J. Steroid Biochem. Mol. Biol.* 41: 231–240, 1992.
24. Oshima, J., M. Watanabe, J. Hirosumi, and H. Orimo. 1,25(OH)₂D₃ increases cytosolic Ca²⁺ concentration of osteoblast cells, clone MC3T3-E1. *Biochem. Biophys. Res. Commun.* 145: 956–960, 1987.
25. Rampe, D., J. M. Caffrey, M. D. Schneider, and A. M. Brown. Control of expression of the 1,4-dihydropyridine receptor in BC₃H₁ cells. *Biochem. Biophys. Res. Commun.* 152: 769–775, 1988.
26. Silve, C. M., G. T. Kradek, A. L. Jones, and C. D. Arnaud. Parathyroid hormone receptor in intact embryonic chicken bone: characterization and cellular localization. *J. Cell Biol.* 94: 379–386, 1982.
27. Van Leeuwen, J. P. T. M., M. P. Bos, C. W. G. M. Lowik, and M. P. M. Herrmann-Erlee. Effect of parathyroid hormone and parathyroid hormone fragments on the intracellular ionized calcium. *Bone Miner.* 4: 177–188, 1988.
28. Wahl, M., M. J. Luccherini, and E. Gruenstein. Intracellular Ca²⁺ measurements with indo-1 in substrate attached cells: advantages and special considerations. *Cell Calcium* 11: 487–500, 1990.
29. Yamaguchi, D. T., T. J. Hahn, A. Iida-Klein, C. R. Kleeman, and S. Muallem. Parathyroid hormone-activated calcium channels in an osteoblast-like clonal osteosarcoma cell line. *J. Biol. Chem.* 262: 7711–7718, 1987.
30. Yukihiro, S., G. H. Posner, and S. E. Guggino. Vitamin D₃ analogs stimulate calcium currents in rat osteosarcoma cells. *J. Biol. Chem.* 269: 23889–23893, 1994.

